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THE RESPONSE OF *PARAMECIUM BURSARIA* TO POTENTIAL ENDOCELLULAR SYMBIONTS [1, 2]

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The series of events leading to experimental endocellular symbioses must begin with ingestion and result in cellular interaction between host and symbiont. In the initial step the feeding mechanism, feeding specificity, and chemoreceptors probably all play some role in screening potential symbionts.

The ability of *Paramecium* and other protozoans to feed selectively has been pointed out by Bragg (1936), Mast (1947), and Nelson (1933).

In the paramecium-alga complex of *Paramecium bursaria* the interacting populations can be separated, recombined and re-combined to give novel combinations (Oehler, 1922; Siegel and Karakashian, 1959; Siegel, 1960; Karakashian, 1963). From these works it can be seen that *P. bursaria* does not exhibit an all-or-none response to foreign algae, but does demonstrate some degree of specificity which is manifest in the size of the intracellular population that develops following "infections."

Also it has been demonstrated that each member of the complex can exist independently of the other (Karakashian, 1963) and therefore each may be considered a facultative symbiont. In addition, the symbiotic complex can be cultured in the absence of an exogenous food supply (Loefer, 1936).

This study was undertaken: (1) to determine the role of the feeding response in establishing an endocellular symbiosis; (2) to test the response of *P. bursaria* to algae of several genera of the chlorococcales representing a spectrum of nutritional types.

Materials and Methods

Stock cultures

The cultures used were obtained from the following sources: *Paramecium bursaria* 32w, the alga isolated from *P. bursaria* 32g (Siegel, 1960), and the food organism *Aerobacter cloacae* were provided generously by Dr. R. W. Siegel and Dr. S. J. Karakashian; *Chlorella vulgaris* 263, *Chlorella variegata* 256, *Trebouxia erici* 912, *Chlorococcum minutum* 117, and *Prototheca zopfii* 328 were obtained from the Indiana Culture Collection (Starr, 1960); the *Chlorella vulgaris* Emerson strain was obtained from Dr. David Appleman.

Culture methods (paramecia)

In general the methods used for the culture of *Paramecium bursaria* were the same as those described by Sonneborn (1950) for the culture of *P. aurelia.*

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The bacterized culture medium was a lettuce infusion inoculated with the washings from a three- or four-day-old bacterial slant and incubated for 24 hours at 25° C before use. All cultures of paramecia were maintained at 25° C on a 12-hour diurnal cycle with a light intensity of 200 foot candles.

Culture methods (algae)

All cultures of algae were maintained on an organic medium described by Loefer (1936) as suitable for the axenic culture of *P. bursaria.*

Experimental methods

For experiments involving the infection of algae into paramecia, the paramecia were taken from a mass culture, centrifuged, and added to a mixture containing 1 ml of freshly bacterized lettuce infusion and 4 ml of the appropriate alga, washed from a 10-day-old agar slant. This procedure yielded cell concentrations as follows: *ca.* 50 paramecia per cc, *ca.* 10^9 bacteria per cc, and from 10^7 to 10^9 algae per cc. These preparations then were allowed to stand under standard culture conditions for the period indicated, at which time a single green paramecium was isolated, washed 4 times in lettuce medium, then removed and allowed to form a mass culture. These isolations were done in triplicate.

Paramecia were counted in a Sedgwick-Rafter counting chamber; algae were counted in a modified Neubauer chamber; bacteria were counted by serial dilutions and emulsion agar plating.

For experiments involving ingestion, 10-ml aliquots were taken from a mass culture of paramecia, and centrifuged for 15 minutes at 2,000 rpm. Starch grains or carmine to be ingested were suspended in bacterized lettuce infusion. In cases where algae were to be ingested, these too were centrifuged and resuspended in bacterized lettuce infusion. The non-living particles or algae or both in bacterized medium were then added to the centrifuged paramecia. After the appropriate length of time, samples were withdrawn and placed within a ring of 5% methylcellulose, cps 15, on a glass slide, and observed under 100 × magnification. Unless otherwise noted 45 animals were observed. Animals were considered positive if they contained even a single particle.

Results

Ingestion

Experiments designed to measure the ingestion of *Chlorella* 32g yielded irregular results. In general great variability was observed in the ability of the paramecia to ingest this alga. Thus it was necessary to determine if the variability was a characteristic pattern in the feeding response of the paramecia or if this variability represented a type of selection on the part of the paramecia.

According to the methods described suspensions of carmine were prepared so as to give concentrations of particles comparable to the concentration of algal cells used, from 10^7 to 10^9 per cc. The paramecia consistently ingested particles of carmine. When placed in these suspensions, they immediately formed many carmine-containing vacuoles. After remaining in these suspensions for 24 hours they con-

tinued to pack themselves with these particles, and presented a striking picture with as many as 8 or 10 bright red vacuoles.

The possibility exists that the variability of ingestion observed in the presence of algae might be explained on the basis of an algal metabolite that inhibits ingestion. To test this possibility suspensions of carmine were prepared in supernatants of 20-day-old algal cultures of *Chlorella* 32g. It was found that the supernatant in no way altered the ingestion of carmine. The paramecia still continue to form many carmine-containing vacuoles.

The particles of carmine used, although not carefully measured, were small enough to exhibit Brownian movement. In contrast to the small size of the carmine particles, the chlorella range in size from 4 to 6 μ. To test the possibility that the carmine particles are being swept passively into the food vacuoles and that the algae are too large to be ingested readily, it was necessary to measure the ingestion of larger particles.

TABLE I

Nutritional types of the algae used

Alga	Nutritional type	Reference
Chlorella sp. (from *P. bursaria*)	facultative heterotroph	Loefer, 1936
Chlorella vulgaris	facultative heterotroph	Algeus, 1948; Van Niel, 1941
Chlorella vulgaris Emerson	obligate autotroph	Finkle *et al.*, 1950; Killam and Myers, 1956; Griffiths, 1961
Chlorella variegata	facultative heterotroph	Fritsch, 1935
Trebouxia erici	facultative heterotroph	Ahmadjian, 1960
Chlorococcum minutum	obligate autotroph	Parker *et al.*, 1961
Prototheca zopfii	obligate heterotroph	Barker, 1935, 1936

Suspensions of Argo corn starch were prepared, and the paramecia mixed with these suspensions. It was observed that the paramecia readily ingested starch grains of all sizes, including particles as large as 14–15 μ.

By a process of crude differential sedimentation, particles of fairly uniform size were obtained by suspending 1 gram of corn starch in a graduate cylinder containing 100 ml of fluid, allowing this to stand for 10 minutes, and then removing 1-ml aliquots from the top layer of fluid. In this manner particles of starch ranging in size from 12–15 μ were obtained. Paramecia were placed in these suspensions, and allowed to feed. Samples were removed and the paramecia were killed with iodine. The paramecia consistently ingested these large particles.

Ingestion of algae

The algae used in these experiments represent a series of chlorococcalean algae of diverse nutritional requirements. These algae are listed in Table I, along with their nutritional type and references to papers where the nutritional requirements of these algae are reported.

Table II summarizes the experiments in which ingestion of these various algae was studied. It can be seen that all of the algae tested, except *Prototheca zopfii,* are ingested to some extent. These data also suggest that the factors involved in

TABLE II

Ingestion of algae by Paramecium bursaria 32w

Particle	Per cent of paramecia ingesting particle
Starch	100
Chlorella 32g	93
C. vulgaris	53
C. vulgaris Emerson	53
C. variegata	47
Trebouxia erici	14
Chlorococcum minutum	27
Prototheca zopfii	0

the ingestion of algae are not the same factors involved in the ingestion of non-living particles. The ingestion of non-living particles is characterized by uniformity, while the ingestion of living particles varies. From observations, it appears that this variation is independent of cell concentration and cell size.

Selection

The above experiments demonstrate the consistency with which paramecia ingest non-living particles. This stands in dramatic contrast to the variation observed when the paramecia are fed algal cells. To further determine the role of the feeding response in the ingestion of algae, experiments were designed to measure the ability of the paramecia to select between different particles when present in approximately equal concentrations.

Paramecia were placed in suspensions of carmine or *Chlorella* 32g or a mixture of carmine and *Chlorella* 32g. These experiments demonstrated that when the algae were present alone, or in a mixture with carmine, they were ingested to a limited extent, while the carmine when present alone or in a mixture was ingested by the entire population of paramecia. A similar experiment was carried out using

TABLE III

Selection between various particles by Paramecium bursaria 32w

Experiment	Per cent of paramecia ingesting particle	Per cent of paramecia ingesting algae
Carmine	100	—
Chlorella 32g	—	46.5
Carmine + *Chlorella* 32g	100	40
Experiment		
Starch	100	—
Chlorella 32g	—	20
Starch + *Chlorella* 32g	100	20
Experiment		
Starch	100	—
Prototheca zopfii	—	0
Starch + *Prototheca zopfii*	100	0

starch grains that approximate algal cells in size, or *Chlorella* 32g or a mixture of the two. The results are similar in that the ingestion of either the starch grains or the algae is independent of the presence of the other particle. This was further confirmed by feeding the paramecia a particle that never was ingested, the colorless alga *Prototheca zopfii.* As in the other experiments this alga was fed alone and in a mixture with a particle that always was ingested, starch grains. In short, the presence of another particle does not alter the ingestion pattern for either particle separately. The results are summarized in Table III.

Clearly these data indicate the ability of the paramecia to ingest a variety of particles, and they reflect the selective ability of *Paramecium bursaria.*

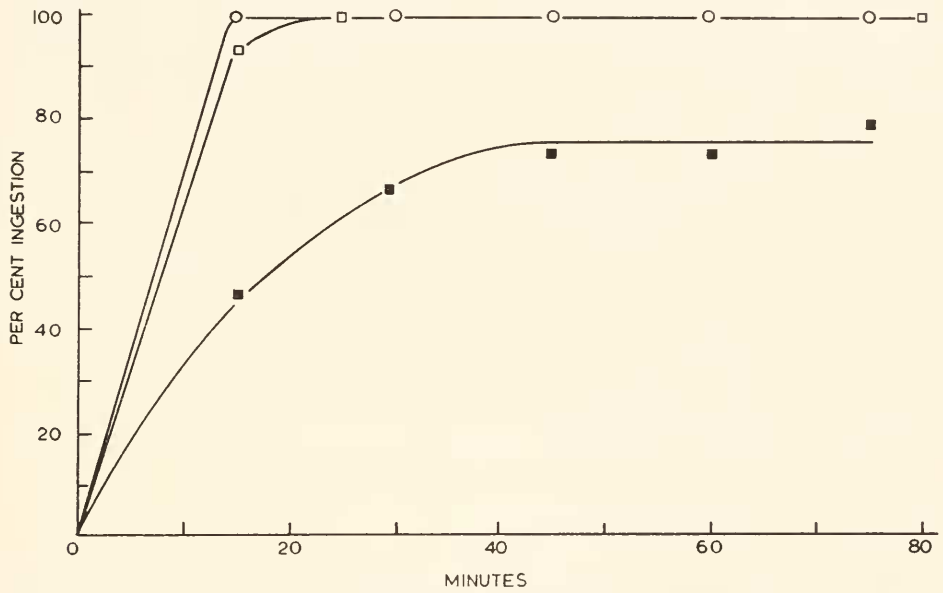

FIGURE 1. The ingestion of light-grown and dark-grown *Chlorella* 32g. Open circles are controls, ingestion of starch grains in the light or in the dark. Open squares of light-grown *Chlorella* 32g. Closed squares are ingestion of dark-grown *Chlorella* 32g. Ordinate is the per cent of paramecia ingesting each particle. Abscissa is time in minutes.

Light and dark experiments

In trying to determine what factors are involved in the ingestion of algae, several chance observations suggested that the paramecia actively ingest the algae at the beginning of a light period.

Based on these observations, the following experiments were undertaken. The algae used were harvested from a twenty-day-old culture of *Chlorella* 32g. Light grown algae were harvested after 105 minutes in the light following a 12-hour dark period. Dark-grown algae were maintained for 15 hours and 45 minutes in the dark prior to use. Dark-grown algae were centrifuged, re-suspended, mixed with paramecia, sampled and killed on a slide, all in the dark to eliminate the possibility

that the paramecia might feed while on the slide when being observed in the light. The paramecia were killed with Patterson's fixing fluid (Zuck, 1959). The dark and light samples were run simultaneously. As controls for these experiments ingestion of starch was measured in the light and in the dark. These paramecia were killed with iodine. In these experiments 30 animals were observed.

These results are summarized in Figure 1. There is no difference in the ingestion of starch in the light or in the dark, and by the time of the first measurement all of the paramecia had ingested starch, and continued to ingest starch for the duration of the experiment. The rate of ingestion of light-grown algae almost equalled the rate of ingestion of starch; the two curves show almost the same steep slope. In conrast, the dark-grown algae at the time of the first measurement were ingested almost 50% less than light-grown algae. At the point where these curves level out, there is approximately a 25% difference between light-grown algae, and the controls.

These data suggest that the paramecia feed selectively and that the feeding mechanism is sensitive to some physiological condition of the alga.

TABLE IV

Percentage of alga-containing progeny descendant from a single infected individual after 13 weeks

Intecting alga[1]	Per cent of progeny that contain the alga
Chlorella 32g	100
Chlorella vulgaris 263	100
Chlorella vulgaris Emerson	61
Chlorella variegata 256	40
Trebouxia erici 912	—
Chlorococcum minutum 117	31
Prototheca zopfii 328	—

Fate of the algae within the paramecia

All of the algae tested except *Prototheca zopfii* are ingested (Table II). However, the fate of the algae varies; some are egested later, apparently undamaged, others are digested visibly, and still others seem to multiply within the paramecia.

Artificial infections and persistence of algae within paramecia

Paramecia containing algae were isolated from mixtures of paramecia and algae that had been allowed to stand for 18 days under standard conditions. No isolations were made in the case of *Trebouxia erici* or *Prototheca zopfii*. These paramecia were isolated, washed four times according to the method described by Sonneborn (1950) and allowed to form mass cultures. The persistence of algae in later generations was studied. The results are listed in Table IV. Although all cultures were started from paramecia that contained algae, only in the case of *Chlorella* 32g and *Chlorella vulgaris* 263 has distribution of algae to all of the paramecia been achieved.

All of the cultures were fed at the same interval to allow for equal growth rates of the paramecia. Therefore, the unequal distribution of algae to the progeny of

paramecia infected with *C. vulgaris* Emerson, *C. variegata,* and *Chlorococcum minutum* might be explained by (1) egestion, (2) digestion, or (3) simply outreproducing the algae. Probably the growth of *Chlorella* 32g and *C. vulgaris* 263 are synchronized with the growth of the paramecia and thus the algae are able to maintain very high intracellular populations.

Discussion

Recent histochemical studies on the food vacuoles of paramecia indicate that functional vacuoles containing non-nutritive particles are formed (Mueller *et al.,* 1965). However, there is no literature specifically relating to the food vacuoles of *P. bursaria.* There is evidence that the feeding responses of many protozoa involve surface reactions: *Balantidium coli* are selective as to quality (Nelson, 1933); *Podophyra collini* demonstrates a preference for ciliates based on an enzyme catalyzed reaction involving the tentacle of the suctorian and the ciliate (Hull, 1961); electron micrographs reveal an initial attachment phase of the particles to the membrane surface during the process of phagocytosis (Brandt and Pappas, 1960). These facts might account for the selective differences in the feeding response of *P. bursaria* to light-grown and dark-grown *Chlorella* 32g, as well as for the failure of paramecia to ingest *Prototheca zopfii.*

Artificial infections

It appears that the relationship between *P. bursaria* and its intracellular algae is neither intimate nor permanent (Table IV), but the infection of *P. bursaria* may depend on the physiological state of the potential symbiont. This is somewhat similar to the infection of *P. aurelia* with kappa particles in that certain stages of the "life cycle" of kappa may be more infective than others (Sonneborn, 1959; Tallan, 1959).

Adaptations of the algae

From the results of this study it appears that various algae, at least under some conditions, are ingested, resist digestion and are capable of growth in the cytoplasm, while only the naturally occurring chlorella and *Chlorella vulgaris* 263 (of those tested) are able to achieve synchronous growth. Perhaps the rapid rate of growth of chlorella serves as a synchronizing mechanism between the paramecium and the algae thereby predisposing the algae to its "parameciumized" fate.

The nature of the interaction

Associations clearly recognizable on a morphological basis as symbioses are widespread and provide not only novel and unique combinations in nature, but are interesting from an evolutionary point of view. Quispel (1951) and Lederberg (1952) have used the term *symbiosis* to describe interactions below the organism level of organization, for example, the interactions between cellular organelles and the cytoplasm.

There is a continuum of interactions that grade between mutualistic symbiosis and parasitism, and the more these relationships are studied, the more it becomes

apparent that these interactions are by no means stable and the balances that exist between host and symbiont can be upset easily (Dubos and Kessler, 1963). To illustrate this, lichens have often been regarded as an example of commensalism but it has been shown that cells of the phycobiont are penetrated by haustoria of the fungus (Moore and McAlear, 1960; Ahmadjian, 1962). Thus it should not be assumed that organisms enter into symbiotic associations for the mutual benefit of each other (Caullery, 1952; Droop, 1963).

Recently, Karakashian (1963) has demonstrated in *Paramecium bursaria* that under certain conditions the algae increase the growth rate of the paramecia. This increase is probably mediated by some photosynthetic product. In addition, if one assumes that under some conditions the development of an intracellular population of algae is also enhanced as a result of the protection afforded the algae in this microhabitat, then, according to the classification of interactions of Burkholder (1952), this relationship might be termed protocooperation. However, these events do not prevail under all conditions. Under stress conditions for either host or symbiont (food limiting, or darkness) the intracellular population will decrease in size (Karakashian, 1963). Under some conditions the algae are egested (Oehler, 1922), and at other times they appear to be digested.

If the alga contributes a photosynthetic product to the complex, then clearly in the dark the interaction will be different from what it is in the light. If in turn the paramecium contributes a carbohydrate to the alga, then here too the interaction will be different in the light and in the dark, since none of the algae, except the obligate heterotroph is dependent on a preformed carbohydrate. Clearly the balances in this relationship are sensitive, and are upset easily. Factors involved must include the physiological condition of the alga, the availability of food for the paramecium, and the intensity of light. Varying any of these conditions alters the interaction between the host and its "plasmid" (Lederberg, 1952).

Karakashian (1963) considers the exchange of metabolites and the alteration of the growth pattern of the symbiotic complex as evidence that the host and its plasmid are a well integrated functioning unit in nature. Muscatine and Lenhoff (1963) have shown that 10% of the carbon fixed by the *Chlorella* of *Chlorohydra viridissima* appears in the host animal. Can the mere exchange of metabolites be taken as evidence for integration between a host and its symbiont? Zabka and Lazo (1962) have shown the reciprocal exchange of radio-phosphorus between a myxomycete, *Fuligo cinera* and an alga, *Chlorella xanthella*, two organisms that do not exist as a symbiotic complex.

In pure cultures of various Chlorophyceae, appreciable amounts of the total carbon fixed appear in soluble form in the external medium. In *Anabaena cylindrica* up to 1.4% of its dry weight is liberated as extracellular pentose (Fogg, 1952). With these works in mind it is not at all surprising to find an exchange of metabolites occurring between the paramecium and the algae, in the *P. bursaria* complex.

It is also necessary to consider whether organisms living together always interact. It is not likely that the encysted flagellates and *P. trichium*, reported by Wenrich (1926), occurring together, interact with each other.

It appears that the relationship between *P. bursaria* and its endocellular symbiont, *Chlorella* sp., typically is ephemeral. The establishment of this relationship

depends on ingestion. The fate of the algae within the paramecium varies. Exchange of metabolites clearly must occur under some conditions, but the actual interactions vary, and can not easily be classified in any conventional system. The interactions are dynamic, and the equilibria are upset easily.

Summary

1. *Paramecium bursaria* ingests a variety of particles, but this ingestion is not random. The paramecia select between different particles of approximately the same size when present in approximately equal concentrations.

2. The paramecia ingested six of the seven species of algae tested. Five of these six are maintained to some extent in the cytoplasm of *P. bursaria.* Maintenance of the alga in the cytoplasm seems independent of the nutritional type of the alga.

3. Of all the algae tested, only the naturally-occurring species of *Chlorella* and a free-living strain of *Chlorella vulgaris* have been established as symbionts, the criterion being the distribution of algae to all of the progeny of the paramecium. The adaptations of the algae to this niche are discussed.

4. The role of (a) selective feeding by the paramecia, and (b) the physiological condition of the alga in establishing this relationship are discussed.

5. The nature of the interaction between *Paramecium bursaria* and its endocellular symbiont is discussed. Apparently this relationship is unusual since it lacks specificity and permanence, two traits characteristic of most symbioses.

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